Control of glycolysis in cultured chick embryo hepatocytes

Fructose 2,6-bisphosphate content and phosphofructokinase-1 activity are stimulated by insulin and epidermal growth factor

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Chick embryo hepatocytes were maintained in monolayer culture in a serum-free chemically defined medium for periods of up to 2 days. Over this time period, insulin provoked selective increases (up to 5-fold) in factors relevant to the control of glycolysis: the activities of phosphofructokinase-1 (PFK-1), phosphofructokinase-2 (PFK-2) and hexokinase isoenzymes and the content of fructose 2,6-bisphosphate (F26BP). Half-maximal effects of insulin on PFK-1 activity were in the physiological range (0.1 nM). Changes in enzyme activities and F26BP content in response to insulin were correlated with stimulation of glycolytic flux as estimated by radioisotopic flux. These data are discussed in relation to known changes which occur in hepatic glycolytic activity and PFK-1 activity in the intact chick around hatching. The effects of insulin on F26BP content, PFK-1 activity and glycolytic flux were mimicked by epidermal growth factor (EGF). In contrast, phorbol esters produced minimal actions on any of the above parameters. Our data indicate that protein kinase C is not involved in the actions of insulin or EGF in control of F26BP content or PFK-1 activity. This work indicates that the related tyrosyl kinase receptors of insulin and EGF may provoke identical responses within hepatocytes, but through the utilization of different transduction systems which merge to common control points.

INTRODUCTION

The molecular mechanism of insulin action remains enigmatic. However, the identification of the protein tyrosyl kinase activity of the insulin receptor and the relationship to the tyrosyl kinase activity exhibited by certain oncogene-encoded proteins and by the receptors for several growth factors, including epidermal growth factor (EGF), suggest that crucial intracellular events may be common to several growth-promoting stimuli [1,2]. Indeed, insulin and EGF can provoke similar responses in cells but, although both hormones may share transduction pathways in part, some aspects may be unique to each hormone. Thus EGF and insulin exhibit several common biological actions on rat liver, but there are responses which are obtained with only one of the hormones [3]. In addition, insulin and EGF exert antagonistic responses in certain liver functions [4].

The development of the embryonic chick proceeds through several defined metabolic stages in which the liver plays a key nutritional homeostatic role; insulin and related growth-promoters may be crucial in the adaptation to the changing nutritional pressures of development. Within the egg, the chick develops in an environment rich in lipid and protein but which lacks carbohydrate. The conversion of gluconeogenic precursors into glucose and the production of ketone bodies from fatty acids and select amino acids by the liver are predominant functions of this tissue in embryonic life [5]. After hatching, the nutrient input for the chick is rich in carbohydrate, and for adaptation to this altered nutritional environment there are dramatic changes in the hepatic enzyme profile [6]. Levels of several lipogenic enzymes increase rapidly in response to hatching and food intake. Changes in plasma insulin concentration parallel the modifications of enzymes around hatching [7]; direct estimates from cell culture studies indicate a crucial role for insulin in provoking a concerted change in the syntheses, and hence

activities, of the group of lipogenic enzymes [8]. The liver is the major site of lipogenesis in avian species and glucose has been implicated as the main direct lipogenic precursor in birds. Thus active lipogenesis would require co-ordinate expression of glycolytic and lipogenic enzyme activities.

We have examined previously the influence of hatching on chick hepatic phosphofructokinase-1 (PFK-1) activity and on fructose 2,6-bisphosphate (F26BP) content; within a period of 1–2 days after hatching there are 2- and 5-fold changes in PFK-1 activity and F26BP content respectively [9]. Within the intact animal, co-ordinate changes in the activities of glycolysis and lipogenesis may be of physiological relevance. The central importance of insulin in the regulation of lipogenic enzymes suggested that this hormone could play a role in co-ordination of PFK-1 activity with the need for provision of glycolytic intermediates as precursors for lipogenesis. In this paper, we describe the use of primary cultures of hepatocytes prepared from embryonic chicks just before hatching in order to examine the hormonal control of PFK-1 activity and related parameters in relation to glycolysis. Our studies indicate a critical role for insulin in regulation of PFK-1 activity and glycolysis and thus in co-ordination of changes of several enzymes within functionally linked metabolic pathways. In addition, EGF, which may be a physiological regulator of liver function, provokes effects similar to those of insulin, indicating that both hormone effector systems may operate through related transduction mechanisms.

MATERIALS AND METHODS

Animals

Fertile chick (*Gallus domesticus*) eggs were obtained from Northern Biologicals, St. Annes on Sea, Lancs., U.K. Eggs were

Abbreviations used: EGF, epidermal growth factor; F16BPase, fructose 1,6-bisphosphatase (EC 3.1.3.11); F26BP, fructose 2,6-bisphosphate; KMB, Krebs–Henseleit buffer; LDH, L-lactate dehydrogenase (EC 1.1.1.27); PDB, 4 β -phorbol 12,13-dibutyrate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PK, pyruvate kinase (EC 2.7.1.40).

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incubated in a conventional forced-draught incubator at 40 °C and at a constant humidity of 60 %.

Reagents

Sources of materials for analysis of PFK-1 activity, and F26BP and ATP contents have been detailed previously [9,10]. ADP, collagenase, lactate dehydrogenase (LDH), myokinase, NADH, NADP⁺, phosphoenolpyruvate (sodium salt), pyruvate and pyruvate kinase (PK) were obtained from BCL, Lewes, East Sussex, U.K. Amberlite CG400, EGF (cell culture grade), fructose 1,6-bisphosphate (F16BP), glucose-6-phosphate dehydrogenase (from *Leuconostoc*), insulin (porcine), oleate (sodium salt), penicillin G, 4 β -phorbol 12,13-dibutyrate (PDB), phosphoglucose isomerase and streptomycin sulphate were purchased from Sigma, Poole, Dorset, U.K. Fungizone was obtained from E. R. Squibb, Hounslow, Middlesex, U.K., and powdered Waymouth medium MB 752/1 was from Gibco, Paisley, Scotland, U.K. D-[3-³H]Glucose and PCS were from Amersham International. All other reagents were of the highest possible grade and were obtained from standard suppliers.

Preparation and culture of hepatocytes

Preparation. Hepatocytes were prepared from 18-day-old chick embryos (i.e. 2–3 days before hatching) by collagenase digestion of the intercellular matrix essentially as described previously [10]. However, precautions were taken to minimize contamination with bacteria and micro-organisms. For example, media [Krebs–Henseleit bicarbonate buffer (KHB) and Waymouth medium] used for cell preparation or culture was either autoclaved or filter-sterilized, where appropriate, before use. In addition, the Waymouth medium used for culture was supplemented with streptomycin (100 mg/l), benzylpenicillin (60 mg/l) and Fungizone (2.5 mg/l).

Culturing of hepatocytes was performed on 35 mm vented culture plates which had previously been precoated with collagen isolated from rat tails. KHB was used for the preparation of hepatocytes as described earlier [10]. After the final centrifugation step in hepatocyte purification, the cell pellet was resuspended in Waymouth medium (containing the additions mentioned above: inoculation medium). Inoculation medium (1 ml) was added to each culture plate and portions (0.25 ml; 2.0–2.5 mg dry weight of cells; 30–40 μ g of DNA) of the final cell resuspensions were added to each culture plate. Plates were placed in a modular incubator and, following gassing with O₂:CO₂ (19:1), the incubator was placed at 37 °C for the period of cell inoculation (3–4 h).

At the end of the period of inoculation the medium was aspirated from adherent cells (75 % of those added, based on DNA content) and these were covered with 1 ml of maintenance medium [this was the same as inoculation medium but was additionally supplemented with oleate (50 mg/ml)–BSA (2 g/l) complex]. As before, the culture plates were incubated at 37 °C for subsequent periods of culture; where necessary, the maintenance medium was replaced after 24 h by a fresh 1 ml portion of the same medium for culture throughout a further 24 h period (during 48 h of culture more than 85 % of cells remained attached).

Hormones and other agents added to cultures were sterilized by filtration and diluted with maintenance medium to the correct final strength for addition (as 10–50 μ l portions) to cell incubations. When maintenance medium was changed during culture, fresh hormones/agents were added to maintain their presence throughout incubations.

Microscopic examination of culture plates during the 48 h of culture indicated that hepatocytes adhered to plates, initially as single cells. At the end of culture the cells retained the morphology

of hepatocytes and, having established cell–cell contacts, they had formed networks of single-cell-thick sheets of cells which effectively covered the majority of the culture plate. Immediately after isolation the cell suspension exhibited an ATP/DNA ratio of 0.27 ± 0.02 nmol of ATP/ μ g of DNA (12 determinations), and throughout the period of culture in Waymouth medium without any additional components, the cellular ATP content was retained at this value (0.27 ± 0.02 and 0.28 ± 0.03 nmol of ATP/ μ g of DNA after the 3 h inoculation period and 48 h of culture respectively; 10 determinations).

Termination of cultures for analytical procedures. In order to terminate incubations, the medium on the culture plate was aspirated and cells retained on the plate were washed once with 1 ml of Hepes-buffered KHB (KHB supplemented with 20 mM-Hepes, pH 7.4) to remove unattached cells. Subsequent treatment of the cell sheet was dependent on the eventual analysis to be performed.

ATP and DNA. For extraction of ATP and DNA, 0.5 ml of 0.25 M-HClO₄ was added to cell sheets. After 10 min on ice, cell extracts were harvested with a rubber policeman, and the plates were re-extracted with a further 0.5 ml of HClO₄. Pooled washings were centrifuged (12000 g, 2 min, room temperature) and 200 μ l of the supernatant was removed and neutralized by the addition of 2 M-KOH containing 0.5 M-triethanolamine (for ATP assay). The remaining supernatant from acid treatment was discarded and the pellet was used for analysis of DNA content.

F26BP. Cells on washed plates were lysed by the addition of 0.25 ml of 50 mM-NaOH. After 10 min cells were harvested as above, and plates were re-extracted with a further 0.25 ml of NaOH. The pooled washings were used for analysis of F26BP.

Enzyme analysis. Cells on plates were removed by the addition of 100 μ l of the appropriate homogenization buffer. Cells were harvested with a rubber policeman, and the plates were re-extracted with a further 100 μ l of the same buffer. The pooled cell extract was subjected to two cycles of freeze–thawing before analysis of enzyme activity. Assays were performed on supernatants produced by centrifugation at 10000 g (2 min at 4 °C).

Analytical procedures

Metabolite analysis. Methods for the analysis of F26BP and ATP have been described previously [9,10]. For determination of F26BP content on large numbers of samples, it was found more convenient to use the microtitre plate assay described by Bruni *et al.* [11]. DNA was quantified by reference to standard DNA (1 mg/ml in 5 mM-NaOH) by the method of Burton [12].

Enzyme assays. Spectrophotometric methods were used for assessment of PFK-1 [9], PFK-2 [9], PK [13], LDH [14], F16BPase [15] and hexokinase [16] activities. Details of extraction buffers and assay conditions are given in the relevant papers. Hexokinase activity was only measured at 100 mM-glucose, and we found it essential to supplement the buffer for PFK-1 extraction with F26BP (20 μ M final concentration) to stabilize PFK-1.

Determination of glycolytic flux. This procedure was based on the protocols described by Hue [17]. As detailed in the legend to Fig. 1, cells were incubated for the appropriate time period in maintenance medium supplemented with 0.5 μ Ci of [3-³H]glucose/ml (giving a final specific radioactivity for glucose in the medium of 18.2 nCi/ μ mol). Reactions were terminated by

addition of 0.1 vol. of 20% (w/v) HClO_4 . Samples were also prepared from cultures at the time of isotope addition for determination of $^3\text{H}_2\text{O}$ background. The entire contents of each HClO_4 -treated extract were then centrifuged (12000 g; 2 min; room temperature) and the supernatants were neutralized with 2 M-KOH containing 0.5 M-triethanolamine. Neutralized extracts were applied to columns (2 cm $\times$ 0.5 cm) of Amberlite CG400 (borate form). The material which flowed through the column and a subsequent 3 ml wash with water were collected for determination of the $^3\text{H}_2\text{O}$ generated during catabolism of the radioactive glucose. Portions (0.25 ml) of this eluant were mixed with PCS and counted for radioactivity.

Statistical analysis and presentation of results

Enzyme activity units are presented as μmol of product formed/min at the specified temperature. Results are given as means $\pm$ S.E.M. for the numbers of independent experiments (i.e. distinct cell cultures) specified. In certain cases the results are presented for single representative experiments; in such cases, the values are means $\pm$ S.D. for the number of individual culture plates for each condition. Significance of differences between treatments was determined by Student's *t* test.

RESULTS AND DISCUSSION

Actions of insulin on PFK-1 activity and other cellular parameters

The PFK-1 activity of cell suspensions immediately after isolation (Fig. 1a) correlates well with the activity determined in the intact liver of 18-day-old embryos [9]. A significant decrease in PFK-1 activity was observed during the inoculation period of culture. Throughout a further culture period of up to 48 h, in the absence of any added factors, a gradual increase was observed in PFK-1 activity. By 48 h of culture the activity, expressed in terms of DNA content, approached that determined for freshly isolated hepatocytes. Assessment of cellular F26BP content indicated that the changes in this metabolite paralleled those seen for PFK-1 activity (Fig. 1c). The gradual increases in F26BP content and PFK-1 activity may be contributory factors towards the enhancement of utilization of $[3\text{-}^3\text{H}]\text{glucose}$ observed throughout the period of culture (Fig. 1d). As an estimate of glycolytic flux, this approach suggests that glycolysis (at least to the level of triose phosphates) is relatively low over the first 12 h in maintenance culture but that this rate increases by 3–4-fold under control incubation conditions alone. Inoculation is

Fig. 1. Effects of insulin, EGF and PDB on the activities of PFK-1 (a) and PFK-2 (b), cellular F26BP content (c) and glycolytic flux (d)

Hepatocytes were prepared and cultured as described in the Materials and methods section. Cells were cultured either in unsupplemented maintenance medium (○) or in the presence of 0.1 μM -insulin (●), 5 nM-EGF (▲) or 1 μM -PDB (■). At the indicated times cultures were terminated and samples were extracted for analysis of PFK-1 activity (a), PFK-2 activity (b), F26BP content (c), and glycolytic flux (d). Methods for analysis are given in the text. For measurement of glycolytic flux (d), maintenance medium was replaced after the desired time of pretreatment (i.e. 0, 12, 24 or 36 h) and replaced with maintenance medium containing $[3\text{-}^3\text{H}]\text{glucose}$ for a further 12 h incubation. This approach generated the assessments of glycolytic flux for the time intervals indicated (i.e. 0–12, 12–24, 24–36 and 36–48 h). The data are from our separate experiments and the values are means $\pm$ S.E.M. The exceptions to this are the 12 and 24 h time points for PFK-2 activity, which are means of two experiments only. In (d): □, control; ■, 0.1 μM -insulin; ▨, 5 nM-EGF; ▩, 1 μM -PDB.

Fig. 2. Effects of insulin and EGF on hepatocyte PFK-1 activity: concentration-dependency

Hepatocytes were prepared and cultured for 48 h as described in the Materials and methods section in the presence of a range of concentrations of insulin (a) or EGF (b). After 48 h of culture, incubations were terminated and samples were extracted for analysis of PFK-1 activity as described in the text. The data are results from a single representative experiment and the values are the means $\pm$ s.d. of triplicate determinations for each condition.

associated with a significant decrease in PFK-2 activity (Fig. 1c). This change may be the causative factor responsible for the decreased F26BP content and PFK-1 activity of hepatocytes at the start of the maintenance period and therefore for the low rate of glycolytic flux determined in the initial stages of culture. Small increases are observed in the activity of PFK-2 throughout culture under control conditions; this change may be the important factor for the increases seen for F26BP, PFK-1 and glycolysis under control conditions.

The presence of insulin ($0.1 \mu\text{M}$) in the maintenance medium produced dramatic stimulations of PFK-1 and PFK-2 activities, F26BP content and glycolytic flux (Fig. 1). By 48 h of culture insulin had produced an approximate 3-fold increase in all parameters. However, when the magnitude of the response of each parameter was studied at earlier time points, it is clear that there is distinct temporal sequence of events. Stimulation of PFK-2 activity and F26BP content was rapid, with significant effects being observed at 12 h of culture. Rapid changes in PFK-2 activity and F26BP content have also been observed in response to insulin in other cell systems [18,19]. Measurement of glycolytic flux indicates the possibility of a biphasic response to insulin. In the first 12 h of culture, insulin provoked a significant but transient stimulation of flux. With subsequent culture the glycolytic flux measured under basal conditions increased, and it is only in the time periods of 24–36 h and 36–48 h that insulin provoked further significant stimulation over the basal rate of glycolysis. Rapid stimulation of glycolysis by insulin has been reported for other cultured cell systems, but no previous data exist on the possibility of a biphasic response to insulin [18,20]. Changes in PFK-1 activity only become notable after 24 h of culture; the relationship of the delayed change in PFK-1 activity and the second-phase stimulation of glycolysis by insulin remains to be examined. In relation to actions on PFK-1 activity in our study, cultured chick embryo hepatocytes exhibited sensitivities within the range of insulin concentrations found under physiological conditions [7]; half-maximal effects of insulin are produced at $(2-4) \times 10^{-10} \text{ M}$ (Fig. 2a).

Further analysis was carried out of the specificity of insulin

action by examination of a series of enzymes involved in glucose metabolism (Table 1). Little change (by comparison with PFK-1) was observed in the activities of PK, LDH and F16BPase (the isoenzyme of PK found in chicken liver, M_2 , is insensitive to dietary or hormonal control [21]). It was notable that insulin produced 3–4-fold increases in the activities of hexokinase isoenzymes. Although early reports suggested that avian species lack hepatic hexokinase isoenzymes with a high K_m for glucose ['glucokinase'] [22], more recent reports have identified a membrane-bound form of glucokinase in chicken liver which is subject to dietary regulation [23,24]. In our present studies we utilized a freeze-thaw technique for enzyme extraction of cellular suspensions which is known to release the membrane-bound form of glucokinase in tissue extracts, and we purposely assayed at high glucose concentrations alone in order to assess total isoenzyme activities. Subsequent experiments would be required to identify the isoenzyme pattern and cellular localization of hexokinase isoenzymes which respond to insulin. However, it may be relevant to point out that studies on the cytosolic

Table 1. Effects of insulin on enzyme activities

Hepatocytes were cultured for 48 h in the presence or absence of $0.1 \mu\text{M}$ -insulin as detailed in the Materials and methods section. Enzyme extraction and conditions for analysis are given in the relevant sections. Results are expressed as munits of enzyme activity/ μg of cell DNA, and the values are the means $\pm$ s.e.m. for four separate experiments. Significance of difference compared with control incubations: * $P < 0.05$.

Enzyme	Activity (munits/ μg of cell DNA)	
	Control	Insulin
PK	0.46 ± 0.11	0.51 ± 0.06
LDH	182 ± 42	258 ± 18
F16BPase	1.04 ± 0.20	0.84 ± 0.11
Hexokinase(s)	0.033 ± 0.009	$0.102 \pm 0.014^*$

glucokinase from rat liver have shown that insulin plays a predominant role in the stimulation of transcription of the gene for glucokinase [25].

Thus, although it is clear that insulin can provoke stimulation of glycolytic flux in chick embryo hepatocytes in primary culture, it is difficult to assess the relative importance of several factors (F26BP, PFK-1, PFK-2, hexokinases) in production of altered metabolic capacity. Our results are in basic agreement with data from other studies with cultured cells indicating that insulin provokes changes in PFK-2 activity and hence F26BP content as an early event [18,19]. As a consequence, glycolytic flux is accelerated. The rapid increase in F26BP content may not only arise as a consequence of an increased PFK-2 activity. The action of glycolytic flux may be secondary to increased cellular contents of hexose monophosphates; insulin stimulation of hexokinase isoenzymes may be of direct significance to increased hexose monophosphate contents. An increase in hexose monophosphates and other regulatory metabolites may in turn influence the activity of PFK-2. Increased hepatic contents of fructose 6-phosphate and glucose 6-phosphate occur within 1–2 days of the transition of chicks from embryonic to neonatal environments (M. J. Hamer, unpublished work). As a consequence, significant stimulation of glycolysis and F26BP content may arise in cultured chick embryo hepatocytes from changes to hexose monophosphate contents, although this remains to be determined.

The molecular level at which insulin stimulates PFK-1 activity has not been addressed in detail in these studies. Inclusion of a transcriptional inhibitor, cordycepin, in cell culture media at a concentration of 2 μM abolished the ability of insulin to stimulate PFK-1 activity after 48 h of culture. At this concentration cordycepin did not modify PFK-1 activity or cell viability (ATP content) in control incubations. These preliminary results indicate that insulin provokes changes at the transcriptional level in order to bring about stimulation of PFK-1 activity. Although this could be interpreted as a direct action on transcription of the gene of PFK-1, our data suggest that, based on the temporal sequence of events that we have measured, there may be a causal relationship between changes in PFK-2 activity and F26BP content with respect to control of PFK-1 activity. Thus changes to the transcription of other genes (PFK-2, hexokinases) may be of significance to a secondary effect on PFK-1 activity. We believe that information obtained by use of cordycepin must be interpreted with caution; inclusion of this agent at higher concentrations ($> 4 \mu\text{M}$) produced cytotoxic actions on cultured chick embryo hepatocytes. Given the possibility of an overall perturbation of cellular function, the conclusions above must be of a preliminary nature and direct confirmation is essential.

Relationship between insulin action and responses to EGF and phorbol esters

EGF (5 nM) produced actions similar to those of insulin on F26BP content, PFK-1 activity and glycolytic flux (Fig. 1). In addition, after 48 h in the presence of EGF, PFK-2 activity was 227.6% of control values (mean of two separate experiments). EGF produced the same relatively rapid and significant stimulation of F26BP content as observed for insulin, with the responses of PFK-1 activity and glycolytic flux being delayed. However, there are slight differences in the time of onset of stimulation of glycolytic flux in response to EGF and insulin. In most cases the maximum effects of EGF were slightly less than those of insulin even when EGF was used at maximally effective concentrations (Fig. 2b). Similar potency differences have been observed for the actions of insulin and EGF on F26BP content of human fibroblasts [26]. Although this response differential may reflect different numbers of EGF and insulin receptors per cell, there may be an underlying difference in the intracellular

Table 2. Effects of various factors on hepatocyte ATP content

Hepatocyte culture and analysis of ATP content are detailed in the Materials and methods section. Hepatocytes were cultured for 48 h either in unsupplemented medium (control) or in the presence of insulin (0.1 μM), EGF (10 nM) or PDB (1 μM). Results are the means $\pm$ S.E.M. of five separate experiments. Significance of difference compared with control incubations: * $P < 0.05$.

Factor	ATP content (nmol/ μg of DNA)
Control	0.178 $\pm$ 0.020
Insulin	0.573 $\pm$ 0.101*
EGF	0.306 $\pm$ 0.078
PDB	0.192 $\pm$ 0.022

transduction factors involved in the response to each agent. In relation to this point, although EGF provokes actions on PFK-1 activity, F26BP and glycolytic flux which are similar to those produced by insulin, we observed different actions on cellular ATP content. In all experiments ATP content was monitored as an assessment of cell quality. Insulin produces a clear increase in cellular ATP content (Table 2). As far as we are aware, no other reports have been presented of regulation of ATP content by insulin in primary cultures of hepatocytes. It is feasible that this is a species- or developmental-stage-specific response. However, the culture medium used for our experiments does not include serum or other added hormones; the presence of such components may mask or even reverse the actions of insulin in other systems. (Despite these comments, there are indications that insulin has a role to play in the normal control of hepatic adenylate or nucleotide contents. Key enzymes of hepatic nucleotide production, and hepatic nucleotide contents themselves, have been reported to be decreased in diabetic rats [27]. Injection of insulin restored values back to normal, if not supranormal. The physiological importance, the causative mechanism and possible counter-regulatory factors involved in this control required further investigation.) by contrast, the actions of EGF on ATP content are relatively weak compared with the response to insulin (Table 2). Thus it is clear from this information that the cellular events triggered by the related receptor kinases of the insulin and EGF receptors are not identical. Despite this, the simultaneous addition of insulin (0.1 μM) and EGF (10 nM) failed to produce an additive effect on PFK-1 activity during an incubation period of 48 h (PFK-1 values as % of control at 48 h: insulin, 296 $\pm$ 31; EGF, 254 $\pm$ 19; insulin plus EGF, 315 $\pm$ 29; single experiment only, values are means of triplicate determinations $\pm$ S.D.). Therefore, even if unique transduction components are used by insulin and EGF, it would appear that the actions of both hormones may depend on a common regulatory mechanism involved in the control of PFK-1 activity.

Insulin-like actions of phorbol esters in cultured and isolated cells have been taken to implicate the involvement of protein kinase C in the transduction pathway for insulin [28]. In chick embryo hepatocyte cultures the phorbol ester PDB produced weak insulin-like effects (Fig. 1). PFK-2 activity after 48 h in the presence of PDB was only 132% of the respective control (mean of two separate experiments). At all time points there were no significant changes in PFK-1 activity or glycolytic flux. After 12 h of culture F26BP content was not altered significantly. By 24 h and 48 h of culture PDB had produced significant increases in cell F26BP content; however, as there were no concomitant significant changes in PFK-1 activity or glycolytic flux, the

physiological relevance of these observations remains unclear. These results contrast with the rapid stimulation of F26BP content of chick embryo fibroblasts by phorbol esters [31]. In addition, PDB failed to produce any change in ATP content (Table 2). PDB (1 μ M) also failed to modify the ability of insulin (0.1 μ M) or EGF (10 nM) to stimulate PFK-1 activity (PFK-1 values as % of control at 48 h: PDB, 166 ± 26 ; insulin plus PDB, 296 ± 24 ; EGF plus PDB, 277 ± 25 ; single experiment only, values are means of triplicate determinations $\pm$ S.D.). The retention of responsiveness to insulin and EGF after prolonged simultaneous incubation with PDB, and the marginal effectiveness of PDB alone, argue against the involvement of protein kinase C in the transduction pathways by which insulin and EGF stimulate PFK-1 activity. However, in the absence of any direct measurement of protein kinase C (or specific isoenzymes), such conclusions can only be preliminary.

Concluding remarks

The information given in this paper indicates that insulin in the absence of any other hormonal factors can provoke a stimulation of the glycolytic capacity of cultured chick embryo hepatocytes. This finding has direct relevance to the adaptation of the chick at hatching to increased availability of glucose from dietary sources, and it links the changes in glycolytic capacity to the enhanced lipogenic capacity of chick liver which is a consequence of hatching. Our previous demonstration of increased PFK-1 activity within the first 2 days of hatching can now be seen to be a consequence of the increase in plasma insulin observed at hatching [7,9].

The mechanism by which insulin provokes stimulation of glycolysis involves a complex network of components. The net response in glycolytic rate could arise from changes in control of enzyme amount (e.g. PFK-1, PFK-2, hexokinases) or from changes in allosteric regulators (e.g. F26BP, ATP). Goodridge has indicated that a major aspect of regulation of lipogenesis by insulin at hatching is through control at the level of synthesis of lipogenic enzymes [29,30]. Previous work from rat liver has suggested that insulin may regulate the rate of PFK-1 degradation [31,32]. Changes in F26BP content may produce stabilization of PFK-1 from degradation [33]. Sequential analysis of the events monitored in this paper may facilitate an enhanced understanding both of the control of PFK-1 and of the relationship to earliest events in the transduction pathway for insulin.

Our studies provide another example of the ability of EGF to mimic the actions of insulin, yet it is clear that there may be subtle differences between the responsiveness to both agents based on responses of ATP content and PFK-1 activity. It is, however, unlikely that either agent in this cell system mediates its actions on PFK-1 activity through the action of protein kinase C. Further analysis of the common and unique events which arise from activation of the receptors for insulin and EGF within the cultured liver cell system described here will represent a valuable system with which to study relationship between glycolytic state of cells and cell transformation.

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